MAQC Study – Data Generation Plan
(*DRAFT*)
Genospectra QuantiGene Platform

Test Site

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Materials Required

Genospectra Products

- QuantiGene kits
- Gene-specific probe sets for over 200 genes

Non-Genospectra Products

- Total RNA, Brain (Ambion)(AB)
- Total RNA, Universal Human Reference RNA (Stratagene) (UHRR)
- Total RNA, Mixture of 75% AB + 25% UHRR
- Total RNA, Mixture of 25% AB + 75% UHRR

Equipment and consumables

- General laboratory equipment include a 96-well plate-based luminometer (Lmax, Molecular Device) and a hybridization incubator (Hybaid) capable of maintaining temperature between 46°C and 53°C.
- General consumables are listed in the QuantiGene assay SOP manual

User Manuals

- Genospectra QuantiGene Assay SOP Manual

Experimental Design

Minimal of 200 genes will be selected for the QuantiGene assay and agreed to by MAQC. The 200 gene will be matched within the ABI's 1,000 gene list, which will be matched within the list of 5,000 RefSeq genes shared by all microarray platforms. The 200 genes will be selected to cover different expression level, fold changes, and some genes showing cross-platform discrepancy in the pilot study.

Agreement need to be reached among Genospectra, ABI, and Stratagene for the platform validation assays. Three, four or five replicate measurements for each gene can be processed for each of the following mRNA samples, depending on the agreement among Genospectra, ABI, and Stratagene:

1. UHRR (100%)
2. AB (25%) vs. UHRR (75%)
3. AB (75%) vs. UHRR (25%)

4. AB (100%)

Total RNA Preparation and Analysis

It is assumed that all four total RNA samples will be provided in aqueous solution ready for use. The total RNA samples will be stored at -80 ^{o}C and will be used directly in the QuantiGene assay, unless there is a need to dilute the sample further. No additional manipulation is required for the QuantiGene assay. As a result, we rely on the quality assessment of the original total RNA samples by Ambion and Stratagene.

QuantiGene® assay conditions:

1. Stock total RNA samples will be diluted to [40ng/ul] in RNase Free water.
2. 100 to 800ng of total RNA will be added to each well of QuantiGene assay, depending on the gene expression level.
3. Hybridization assay will be carried out per QuantiGene® manual.
4. Background signal for each gene will be assessed in a QuantiGene assay with no sample RNA added, just the probe set.

Data Acquisition and Analysis

QuantiGene assay signal is measured by a luminometer, and the Relative Luminescent Unit (RLU) data will be directly read from the luminometer machine. The data will be exported into an Excel file and reported directly to MAQC.

For those genes (e.g. selected genes from MAQC pilot II study) whose expression in AB and UHRR is substantially different (e.g. over 100: 1 ratio), we expect an assay signal ratio of 100: 75:25:0 ratio for the four total RNA samples. The closeness to the expected 100:75:25:0 ratio will be a measurement of the assay platform accuracy.

Once the assay platform accuracy is established, the relative ratio of the 200 genes among different samples will be assessed, and their correlation with the data from other platforms will be compared.

Overview of the QuantiGene Workflow

Day One:

A. Prepare the QuantiGene® Reagents and gene-specific Probe Set

B. Prepare RNA samples for the selected genes.

C. Follow Experimental Layout on Plate Map.

Day Two:

D. Prepare the QuantiGene Reagents used for the target mRNA.

E. Amplify the Signal and Detect target mRNA.

F. Analyze the Data.

QuantiGene Assay SOP

PURPOSE:

This protocol describes the procedure for performing, single-plex QuantiGene assays with purified RNA using the LMAX luminometer.

SCOPE:

This procedure applies to all R&D personnel performing a QuantiGene single-plex assay using purified RNA or IVTs as the sample source.

KEY SPONSOR AND RESPONSIBILITY:

The R&D Manager is responsible for the content, updating and implementation of this document.

EQUIPMENT:

1. Pipettes, single and multichannel
2. Vortex mixer
3. LMAX luminometer
4. Incubators: 46° +/-1.0°C and 53° C +/- 1.0° C
5. Quick spin bench top micro-centrifuge

MATERIALS

1. QuantiGene Assay Kit (at 4°C - capture plates, amplifier, label probe, substrate; at 15-30°C - amplifier/label probe diluent, lysis mixture)
2. Plastic pipette tips, aerosol resistant
3. Disposable reagent reservoirs, 25 and 100 mL capacity
4. Plate Sealers (Bioexpress, Cat. No. T-3025-8B)
5. 10X or 1X Wash Buffer
6. 10% Lithium Lauryl Sulfate solution
7. Capture Buffer
8. 1 mg/mL tRNA (Blocking Agent)
9. Purified total RNA, mRNA, or IVT RNA
10. Appropriate Probe Set(s) with pooled Label Extenders, pooled Capture Extenders and pooled Blocking Probes
11. Nuclease Free water

SOP Procedure:

Day One:

Step	Action
1	Bring the **Capture Plate** and target-specific probes to room temperature.
2	Check to be sure the **Lysis Mixture** does not have precipitates. If you observe precipitates, then perform the following: a. Place the bottle in a 37ºC water bath. b. Gently swirl contents occasionally until all precipitates dissolve.
3	Prepare the **Concentrated Probe Sets** for the target RNA of interest by adding 50 µl each of pooled 1X CE, (Capture Extender, 50 fmol/µl), 1X LE, (Labeled Extender, 200 fmol/µl), and 1X BL, (Blocking Probe, 100 fmol/µl), probes to 850 µl of **Lysis Mixture** (from QuantiGene kit). **Note: This preparation of concentrated probe set is enough for one 96-well plate.**
4	Prepare **Lysis Working Reagent**: Add 15 µl of yeast tRNA, to 6 ml of **Lysis Mixture**
5	Depending upon the expected abundance of the specific mRNA to be measured, dilute the RNA sample with **Lysis Working Reagent** (see Step 4, above) to a final concentration of 0.02 to 2 µg/10 µl, if using mRNA dilute to a final concentration of 0.4 to 40 ng/10 µl. **Note:** For a gene with a moderate level of expression, 0.2 µg/10 µl or 4 ng/10 µl of mRNA are recommended.
6	Dispense 50 µl of **Capture Buffer,** (Catalog # QGR-004-002), to the **Capture Plate** well
7	Dispense 40 µl of **Lysis Mixture** to the **Capture Plate** well.
8	Dispense 10 µl of **RNA sample** to the **Capture Plate** well.
9	Dispense 10 µl of **Concentrated Probe Sets** to the **Capture Plate** well.
10	Seal the **Capture Plate** with a plate sealer. Incubation the sealed plate at 53°C for 16-20 hours.

Day Two:
Procedural Guidelines

- We recommend preparing the **Substrate**, **Amplifier** and **Label Probe** immediately before use. Prior to use, bring these reagents to room temperature.
- Vortex and briefly centrifuge the **Amplifier** and **Label Probe** to bring the contents to the bottom of the tube before use.
- Calculations below for the **Wash Buffer** include a 20% overage. Calculations for the **Amplifier, Label Probe and Substrate Working Reagents** include a 10% overage. Determine the specific volume requirements for your system.

D. Prepare the Reagents

Step 1

Prepare the **Wash Buffer**:

a. Combine the reagent volumes appropriate for the number of plates used in the assay.
b. Stir gently to mix.
c. Keep mixture at room temperature.

Capture Plates	10X bDNA Wash Buffer	Distilled H_20	Total Volume
1	40 ml	360 ml	400 ml
2	80 ml	720 ml	800 ml

Step 2

Prepare the **Amplifier Working Solution**:

a. Combine the reagent volumes appropriate for the number of plates used in the assay.
b. Stir gently to mix.
c. Keep mixture at room temperature.

Capture Plates	Amplifier/Diluent	Amplifier
1	11.6 ml	11.6 µl
2	22.0 ml	22.0 µl

Step 3

Prepare the **Label Probe Working Reagent:**

a. Combine the reagent volumes appropriate for the number of plates used in the assay.
b. Stir gently to mix.
c. Keep mixture at room temperature.

Capture Plates	Amplifier/Diluent	Label Probe
1	11.6 ml	11.6 µl
2	22.0 ml	22.0 µl

Prepare the Reagents (cont.)

Step	Action
4	Prepare the **Substrate Working Reagent**: a. Combine the reagent volumes appropriate for the number of plates used in the assay. b. Stir gently to mix. It is normal for the **Substrate Working Reagent** to become turbid after the addition of the **10% Lithium Lauryl Sulfate**. c. Cover the solution with aluminum foil and keep at room temperature.

Capture Plates	Substrate	10% Lithium Lauryl Sulfate
1	11.6 ml	34.8 µl
2	22.0 ml	66.0 µl

E. Amplify and Detect the Target mRNA

Step	Action
1	Wash the **Capture Plate:** a. Remove the plate from the incubator and remove the seal from the plate. b. Add **250 µl Wash Buffer** to the wells then follow one of the methods below.

Centrifugation Method [Highly recommended]	Aspiration Method
a. Decant the **Wash Buffer** and **Cell Lysate** to a sink. b. Wash each well 3 times with 350 µl of **Wash Buffer.** c. Decant the buffer to a sink and keep plate face down on paper towels. d. Following third wash use **centrifugation** to remove traces of the **Wash Buffer** from the plate. Lay the plate face down on 2 to 3 paper towels and put in the centrifuge bucket. Spin at 240 g for 1 minute.	a. Aspirate the **Wash Buffer** and **Cell Lysate.** b. Wash each well 3 times with 350 µl of **Wash Buffer.** c. Completely remove **Wash Buffer** from the wells; the aspiration nozzle may be lowered down the side of the well until it contacts the bottom. **Notes:** **1. Try not to touch the bottom of the well.** **2. Try to touch the same corner of the well with the pipette tip every time.**

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Wash the Capture Plate:

c. Remove the plate from the incubator and remove the seal from the plate sealer.

d. Add 250 µl Wash Buffer to the wells then follow one of the methods below.
<table>
<tr><th>Spin Approach
[Highly recommended]</th><th>Aspiration
Approach</th></tr>
<tr>
<td>e. Decant the Wash Buffer and Cell Lysate to a sink.
f. Wash each well 3 times with 350 µl of Wash Buffer.
g. Decant the buffer to a sink and keep plate face down on paper towels.
h. Following third wash use spinning to remove traces of the Wash Buffer from the plate. Lay the plate face down on 2 to 3 paper towels and put in the centrifuge bucket. Spin at 240 g for 1 minute.</td>
<td>d. Aspirate the Wash Buffer and Cell Lysate.
e. Wash each well 3 times with 350 µl of Wash Buffer.
f. Completely remove Wash Buffer from the wells; the aspiration nozzle may be lowered down the side of the well until it contacts the bottom.
Notes:
3. Try not to touch the bottom of the well.
4. Try to touch the same corner of the well with the pipette tip every time.</td>
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Amplify and Detect the Target mRNA (cont.)

Step	Action
2	Immediately add 100 µl of **Amplifier Working Reagent** to each well.
3	Reseal and incubate the plate at 46º to 53ºC for 60 minutes.
4	Wash the **Capture Plate**: a. Remove the plate from the incubator and remove the seal. b. Immediately aspirate or decant the **Amplifier Working Reagent** from the wells and wash each well three times with 350 µl of **Wash Buffer**. c. Completely remove **Wash Buffer** from the wells. (Refer to Step 1.)
4	Wash the **Capture Plate**: d. Remove the plate from the incubator. e. Immediately aspirate the **Amplifier Working Reagent** from the wells and wash each well three times with 350 µl of **Wash Buffer**. f. Completely remove **Wash Buffer** from the wells. (Refer to Step 1.)

5	Immediately add 100 µl of **Label Probe Working Reagent** to each well.
6	Reseal and incubate the plate at 46º to 53ºC for 60 minutes.
7	Wash the **Capture Plate**: a. Remove the plate from the incubator and remove the seal. b. Immediately aspirate or decant the **Label Probe Working Reagent** from the wells and wash each well three times with 350 µl of **Wash Buffer**. c. Completely remove **Wash Buffer** from the wells. (Refer to Step 1.) **Note: After hybridizing with Label Probe do not let the plate dry for more than 5 minutes to avoid partial inactivation of the AP activity.**
7	Wash the **Capture Plate**: d. Remove the plate from the incubator. e. Immediately aspirate the **Label Probe Working Reagent** from the wells and wash each well three times with 350 µl of **Wash Buffer**. f. Completely remove **Wash Buffer** from the wells. (Refer to Step 1.) **Note: After hybridizing with Label Probe do not let the plate dry for more than 5 minutes to avoid partial inactivation of the AP activity.**
8	Immediately add 100 µl of **Substrate Working Reagent** to each well.
9	Reseal and incubate at 46º to 53ºC for 30 minutes.
10	Remove the plate from the incubator and transfer the plate to a luminometer (without a plate heater) and allow the plate to cool for 5 minutes. **Alternatively**, for a luminometer with a plate heater, allow the plate to equilibrate in the tray for 5 minutes.
11	Measure the luminescence of each well.